IDENTIFICATION OF A SECOND β CHAIN IN PIG BRAIN TUBULIN

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1. Introduction

Microtubules are the main structural components of cilia, flagella and the mitotic apparatus. They also participate in morphogenesis, cell orientation, intracellular transport and secretion processes [1]. The building block of microtubules, the protein tubulin, is generally assumed to be a heterodimer of α and β chains, each of mol. wt ~55 000 [2]. Recently, however, a third component has been found in purified tubulin preparations from both Ehrlich ascites tumour cells and pig brain which migrates between the α and β tubulins on SDS–polyacrylamide gel electrophoresis under given conditions [3]. It is possible that this protein represents a specific component of the microtubule system.

Here, the third tubulin component in a preparation of tubulin from pig brain has been identified as a second β chain. An improved procedure for the preparative isolation of tubulin α and β chains is described, and the tubulin subunits have been characterised by limited proteolysis and peptide mapping.

2. Methods

2.1. *Preparation of tubulin*

Tubulin was isolated from pig brain by chromatography of a 100 000 × *g* supernatant on DEAE-cellulose similar to the methods in [2,4] employing a linear 0.1–0.3 M NaCl gradient [5]. It was identified by the fluorescence of its complex with colchicine [6]. The preparation was assayed for impurities by disc gel electrophoresis in the system of [7] using 8% gels.

Protein concentrations were determined by the Lowry method [8].

Estimations of RNA and DNA content were made by the method in [9].

2.2. *Isolation of tubulin subunits*

The isolation of the tubulin subunits was based on the method in [10]. About 300 mg tubulin reduced with mercaptoethanol and alkylated with iodoacetic acid [11] was incubated in 1% sodium dodecyl sulphate (SDS) and 0.01 M sodium phosphate (pH 6.4) at 60°C for 30 min. It was then applied to a 2.5 × 70 cm column of hydroxylapatite equilibrated at 30°C with 0.01 M sodium phosphate (pH 6.4) and 0.1% SDS. After washing the column with 200 ml equilibration buffer, it was developed with a linear gradient (1.5 × 1.5 l) of 0.2–0.4 M sodium phosphate (pH 6.4) containing 0.1% SDS. The various fractions were dialysed overnight against 20 vol. distilled water at room temperature followed by dialysis against 3 changes of 20 vol. 1 mM ammonium hydrogen carbonate for 3 days at 4°C. Tubulin subunits were identified in the Laemmli disc gel electrophoresis system [12] using 7.5% gels and an acrylamide:*N,N'*-methylene-bis-acrylamide ratio of 37.5:1.

2.3. *Peptide mapping*

Enzymatic digestions with *Staphylococcus aureus* V8 protease (Miles Labs.) in a 5% acrylamide spacer gel and electrophoresis on 15% acrylamide gels were performed as in [13]. Chymotryptic and tryptic digests were also resolved on this electrophoresis system after incubation of tubulin subunit preparations containing 1 mg protein/ml, 0.5% SDS, 10% saccharose and 5 mM Tris–glycine buffer (pH 8.3) with 10 μg/ml enzyme for 2 h at 25°C.

Proteins and peptides on electrophoresis gels were

stained with a solution containing 0.034% Coomassie blue R-250, 45% methanol and 9% acetic acid. The gels were destained with a solution containing 10% methanol and 15% acetic acid. Estimations of the amount of each stained protein were made by scanning the gels with a Vernon scanner.

3. Results and discussion

3.1. *Isolation of tubulin subunits*

Separation of tubulin subunits is shown in fig.1. The small peaks at the beginning of the salt gradient represent RNA, which was found to account for ~2% by wt of the material in preparations of tubulin isolated from DEAE-cellulose. DNA was not detected. The minor tubulin component elutes slightly later than β-tubulin. It is unlikely that this component is an artefact because preparations of tubulin always contained about the same amount of minor component (14%), α tubulin (50%) and β tubulin (36%) as measured by gel scanning. Furthermore, tubulin prepared by a quite different method also contained this component [3]. On the electrophoresis system shown in fig.2, the minor component migrates at the same rate as β tubulin, in spite of the wide separation of the α and β bands. This system has been used for the

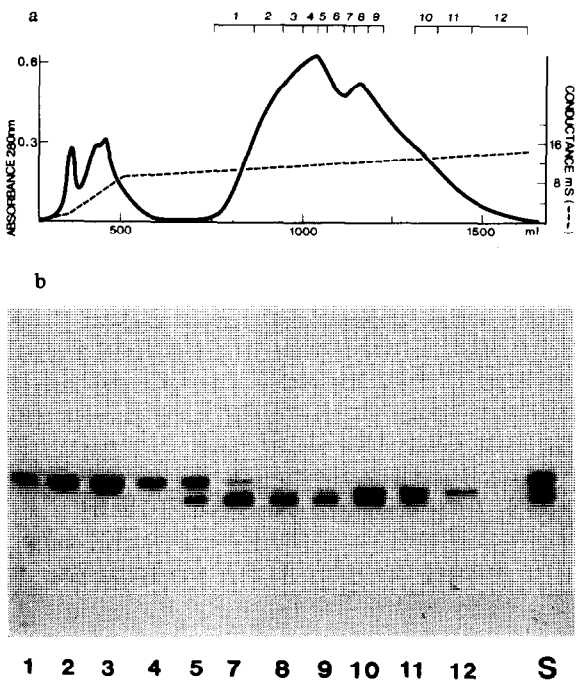

Fig.1. (a) Elution of tubulin subunits from hydroxylapatite. Carboxymethylated tubulin (300 mg) incubated with 1% SDS and 0.01 M sodium phosphate (pH 6.4) for 30 min at 60°C was applied to a 2.5 × 70 cm column of hydroxylapatite equilibrated with 0.1% SDS and 0.01 M sodium phosphate (pH 6.4) at 30°C. The subunits were eluted with a linear gradient (1.5 × 1.5 l) of 0.2–0.4 M sodium phosphate (pH 6.4) containing 0.1% SDS. (b) SDS–polyacrylamide slab gel electrophoresis of the tubulin subunit fractions eluted from hydroxylapatite. System of Laemmli [12] using a 7.5% gel and an acrylamide:*N,N'*-methylenebisacrylamide ratio of 37.5:1. S is the tubulin starting material.

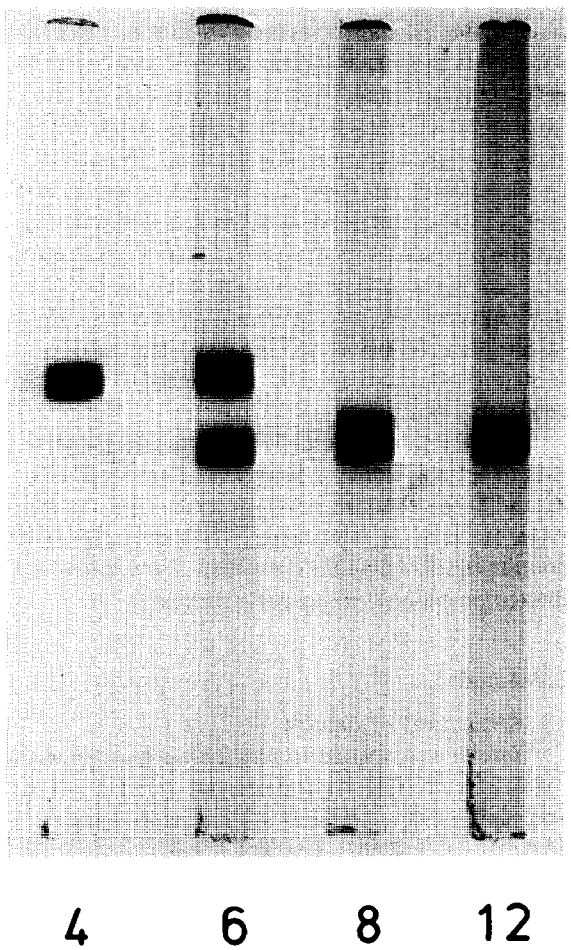

Fig.2. Migration of tubulin subunits in the SDS–polyacrylamide gel electrophoresis system [7] employing 8% gels. Numbers represent the tubulin subunit fractions of fig.1.

preparative isolation of the α and β tubulin subunits from chick brain and from the flagella of sea urchin sperm [14]. The major differences of the electrophoresis system shown in fig.2 from that in fig.1 are a higher concentration of Tris–glycine (4:1) and a lower concentration of SDS (1:3.3) in the electrode buffer, and the absence of SDS in the running gel.

3.2. *Peptide mapping*

The proportion of minor component in preparations of tubulin (combined amounts of minor component and β tubulin equal that of α tubulin), its elution behaviour on hydroxylapatite, and its migration with β tubulin in an electrophoresis system in which the α and β bands are widely separated, suggested that it was also a β tubulin. This was indeed shown to be the case by comparing the peptide maps of fractions 2, 8 and 12 of fig.1, containing purified α tubulin, β tubulin and minor component, respectively (fig.3,4). The peptide patterns for α tubulin are quite different from those of the other two subunits. In contrast, the peptide maps of β tubulin and of the minor component are very much alike, but not identical. The kinetics of the digestion of α tubulin with *Staphylococcus aureus* V8 protease are also quite distinct from those of the other subunits. Whereas α tubulin and its larger breakdown products are very quickly digested, the other subunits and their larger cleavage products are digested more slowly, resulting in an even distribution of large, medium and small peptides. Proteolysis with chymotrypsin is very slow for all the subunits, and a large proportion of each remains undigested. The digestion with trypsin, on the other hand, under exactly the same conditions as for chymotrypsin, proceeds much faster. Only a very small amount of each subunit is still intact. Trypsin, which cleaves specifically on the carboxyl-terminal side of lysine and arginine, has a quite different specificity from *Staphylococcus aureus* V8 protease, which cleaves

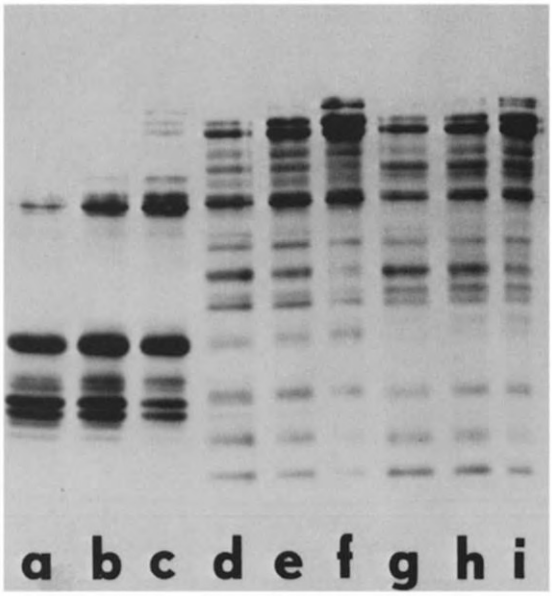

Fig.3. SDS–polyacrylamide gel electrophoresis of *Staphylococcus aureus* V8 protease digest of α tubulin, β tubulin and minor tubulin component. Acrylamide gel (15%): 35 μg of α tubulin (a–c), β tubulin (d–f) and the minor tubulin component (g–i) incubated with 0.15 μg, 0.10 μg and 0.03 μg enzyme (from left to right) in the spacer gel for 10 min.

Fig.4. SDS–polyacrylamide gel electrophoresis of the chymotryptic and tryptic digests of α tubulin, β tubulin and minor tubulin component. Acrylamide gel (15%): 35 μg enzyme digest applied/sample well. Tubulin subunit (1 mg/ml) in 0.5% SDS, 10% saccharose and 5 mM Tris–glycine buffer (pH 8.3) was incubated with 10 μg/ml enzyme for 2 h at 25°C. (a–c) Chymotryptic digest of α tubulin, β tubulin and minor tubulin component, respectively; (d–f) tryptic digest of α tubulin, β tubulin and minor tubulin component, respectively.

specifically on the carboxyl terminal side of glutamic and aspartic acids [15]. Thus, it seems more probable that the differences between the peptide maps of the two β tubulins arise from differences in the amino acid sequences rather than from the modification of a particular amino acid.

A comparison of the tubulin subunits from pig brain and Ehrlich ascites tumour cells, isolated from polyacrylamide electrophoresis gels, has also shown that the minor component appears to be similar but not identical to β-tubulin (K.H. Doenges, personal communication).

Investigations on the primary structure of tubulin subunits should be facilitated by the procedure for their isolation described here. About 90 mg α tubulin and 70 mg β tubulin were obtained from 300 mg of tubulin in a single chromatographic step on hydroxylapatite.

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